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Evidence of oxidative stress in the circulation of ovarian cancer patients

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Abstract

Background: Ovarian cancer is the leading cause of death due to gynecological malignancies among women. The extent of free radical induced oxidative stress can be exacerbated by the decreased efficiency of antioxidant mechanisms. The present study was conducted to investigate the extent of oxidative stress and the levels of antioxidants in the circulation of ovarian cancer patients. *Methods*: Plasma thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) and the levels of antioxidants such as superoxide dismutase (SOD), catalase (CAT), vitamin C and vitamin E were estimated in the circulation of 30 ovarian cancer patients and an equal number of age-matched normal subjects as control. *Results*: Significantly increased concentrations of plasma TBARS and CD and significantly lowered levels of SOD, CAT, vitamin C and vitamin E were observed in ovarian cancer patients as compared with normal subjects. *Conclusion*: The low levels of SOD, CAT, vitamin C and vitamin E in the plasma of ovarian cancer patients may be due to their increased utilization to scavenge lipid peroxides as well as their sequestration by tumor cells. Increased levels of lipid peroxidation may be due to excessive oxidative stress caused by incessant ovulation or epithelial inflammation.

Keywords: Antioxidants; Free radicals; Ovarian cancer; Oxidative stress

1. Introduction

Ovarian cancer is the leading cause of death due to gynecological malignancies and is the fifth most common cause of mortality from cancers among women [1]. In India, 15% of all gynecological cancers is ovarian malignancy [2] and it represents the greatest clinical challenge. Risk factors for ovarian carcinoma include inflammation, excessive number of life time ovulations, increases in steroid hormone levels, heredity, infertility, oral contraceptive pills, age, asbestos, talc and reproductive factors such as nulliparity [3,4]. Ovarian cancer at an early stage is asymptomatic, but later the main symptoms include abdominal swelling, bloating, pain and pressure [5]. Recent molecular studies have shown that ovarian cancer has acquired genetic alterations of oncogenes and tumor suppressor genes such as BRCA1, p^{53}, nm23 and K-ras, which may be due to inflammation and oxidative stress [6].

Oxidative stress is potentially harmful to cells and reactive oxygen species (ROS) are known to be induced in the initiation and progression of cancer

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[7]. ROS can damage cellular components such as lipids, proteins and DNA, affecting enzyme activity and membrane function [8]. Humans are well endowed with enzymic and non-enzymic antioxidants such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione, ascorbic acid (vitamin C) and alpha-tocopheral (vitamin E) [9]. Under conditions of excessive oxidative stress, however, cellular antioxidants are depleted [10].

In recent years, there has been a growing interest in studying the role played by lipid peroxidation and antioxidants in ovarian cancer patients [11,12]. Therefore, the aim of our study was to assess the lipid peroxidation as indicated by TBARS and conjugated dienes and antioxidants such as SOD, CAT, vitamin C and vitamin E in circulation of women with ovarian cancer and to compare our findings with age-matched controls.

2. Materials and methods

2.1. Subjects

Thirty newly diagnosed ovarian cancer patients who obtained a transvaginal ultrasonography (USG) at Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar, Tamil Nadu, India, and who had not undergone any previous treatment for their tumors were selected for the study. The mean age of the patients were 58.6 ± 7.3 years with a range of 50–70 years. Thirty healthy volunteers (all women 58.2 ± 7.6 years; 50–70 years) served as control subjects. Oral consent was obtained from both the ovarian patients and the normal subjects before the study. The present study was approved by the Human Ethical Committee, Rajah Muthiah Medical College and Hospital, Annamalai University, Tamil Nadu, India.

Transvaginal ultrasonography (USG) which allows visualization of both the ovaries was the screening basis for identifying controls versus ovarian cancer patients. Ovarian volume was calculated by means of formula $L \times H \times W \times 0.52$. An ovarian volume ≥ 20 cm^3 in premenopausal women and ≥ 10 cm^3 in postmenopausal women was considered abnormal. Ovarian tumors showed irregular wall, multiple septations, heterogenicity and ascites. Ovarian carcinoma was subsequently confirmed by study in the histology of biopsy specimen patients with mucinous cystadenocarcinoma and papillarycystadenocarcinoma were only included in our study. Patients with benign appearing 'cyst' or 'endometriotic cyst' were excluded from our study. Ovarian carcinomas were staged according to the classification of International Federation of Gynecology and Obstetrics (FIGO) and all the patients examined were categorized as either stage II or IIc. All the patients and control subjects were free from any other gynecologic malignancies such as the breast, cervical and uterine cancer and also any concomitant illness such as diabetes mellitus and liver disease. Control subjects were further screened as the subset of women who by history were free of any prior gynecologic disease or known dysfunction and had normal USG findings and pelvic examination.

USG findings were reviewed in a standardised manner by staff radiologists at Rajah Muthiah Medical College and Hospital.

2.2. Sample collection

Blood was collected by venous arm puncture from the ovarian cancer patients preoperatively in heparinized tubes and the plasma separated by centrifuging at $1500 \times g$ for 10 min. After separating the plasma, the packed cells (RBCs) were washed thrice with cold physiological saline. To determine the activity of RBC antioxidant enzymes, hemolysate was prepared by lysing a known volume of erythrocytes with cold hypotonic phosphate buffer, pH 7.4. The hemolysate was separated by centrifuging at $2500 \times g$ for 10 min at 2 °C. Biochemical estimations were carried out immediately.

2.3. Biochemical estimations

Lipid peroxidation was estimated by the measurement of thiobarbituric acid reactive substances (TBARS) in plasma by the method of Yagi [13]. Plasma was deproteinized using 0.083 N sulfuric acid and 10% phosphotungstic acid and centrifuged at $3000 \times g$ for 10 min. The sediment was treated in a similar manner again with 0.083 mol/l sulfuric acid and 10% phosphotungstic acid. Finally, the sediment was suspended in distilled water followed by thiobarbituric acid–acetic acid reagent and heated at 95 °C

Table 1
Circulating TBARS and conjugated dienes levels in ovarian cancer patients and normal subjects (mean ± S.D.; $n = 30$)

	Control ($n = 30$)	Patients ($n = 30$)
Plasma TBARS (nmol of MDA formed/ml of plasma)	2.13 ± 0.18 [2.76–3.99]	5.64 ± 0.52*
Conjugated dienes (μmol/ml of plasma)	0.71 ± 0.06 [0.6–1.2]	1.71 ± 0.13*

Values in square brackets indicate the normal reference range.
* $P < 0.001$ as compared with normal.

for 60 min. After cooling, the pink color was extracted in *n*-butanol, and measured using spectronic 20 colorimeter at 535 nm. 1,1′,3,3′-Tetramethoxypropane was used as standard.

Conjugated dienes were estimated by the method of Rao and Recknagel [14]. Conjugated dienes were extracted from the plasma using chloroform–methanol reagent (2:1 v/v) and centrifuged at 1500 × *g* for 5 min. The extract was then treated with cylcohexane and the absorbance was read at 233 nm against cyclohexane blank. The amount of conjugated dienes formed was calculated using a molar extinction coefficient of 2.52×10^4 cm^{-1}.

Superoxide dismutase (SOD, EC 1.15.1.1) was assayed by the method of Kakkar et al. [15]. The enzyme was extracted from the hemolysate using ethanol–chloroform (2:1) mixture. An aliquot of the supernatant was treated with 0.1 ml phenazine methosulfate (185 μmol/l), 0.3 ml nitroblue tetrazolium (300 μmol/l) and 0.2 ml reduced nicotinamide adenine dinucleotide (780 μmol/l). The reaction was arrested by adding glacial acetic acid for 60 s. The color which developed after the extraction with butanol was read at 560 nm. The enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard conditions were taken as 1 enzyme unit. A system devoid of the enzyme served as the control.

Catalase (CAT, EC 1.11.1.6) activity was assayed by the method of Sinha [16]. Hemolysate was treated with H_2O_2 (0.2 mol/l) and the reaction was arrested after 60 s by the addition of dichromate–acetic acid reagent, cooled and the intensity of color read at 620 nm. Various aliquots of H_2O_2 were used as the standard. A system devoid of the enzyme served as the control.

Ascorbic acid (vitamin C) was estimated by the method of Roe and Kuether [17]. Plasma was deproteinized using trichloroacetic acid. Acid-washed norit was added to the supernatant and filtered. This converts ascorbic acid to dehydro ascorbic acid. Dinitrophenylhydrazine was added to the filtrate and incubated for 3 h at 37 °C. Ice-cold concentrated sulfuric acid was added, mixed well and the color read at 520 nm after 30 min.

Vitamin E was estimated by the method of Baker et al. [18]. Plasma was treated with petroleum ether and ethanol, mixed and centrifuged at 1000 × *g* for 10 min. Dipyridyl and ferric chloride were added to the supernatant and the mixture kept in the dark for 5 min. The color developed was read at 520 nm.

Hemoglobin in the hemolysate was measured according to the method of Drabkin and Austin [19]. Blood was diluted in an alkaline medium containing potassium cyanide and potassium ferricyanide (Drabkin's reagent). Hemoglobin oxidized to methemoglobin combines with cyanide to form cyanmethemoglobin which was measured at 540 nm.

The biochemical data are expressed as mean ± S.D. Statistical significance was analyzed using Student's *t*-test.

3. Results

Table 1 shows the level of lipid peroxidation in plasma of normal and ovarian cancer patients. Lipid peroxidation as assessed by TBARS level was significantly higher in ovarian cancer patients as compared

Table 2
Circulating antioxidant levels in ovarian cancer patients and normal subjects (mean ± S.D.; $n = 30$)

	Control ($n = 30$)	Patients ($n = 30$)
SOD ([a]Units/mg Hb)	1.91 ± 0.29 [2–4]	0.9 ± 0.11*
Catalase ([b]Units/mg Hb)	5.71 ± 0.61 [6–8]	4.4 ± 0.39*
Vitamin C (mg/dl of plasma)	1.05 ± 0.09 [0.6–1.2]	0.40 ± 0.03*
Vitamin E (mg/dl of plasma)	2.82 ± 0.20 [2–3]	1.36 ± 0.10*

Values in square brackets indicate the normal reference range.
[a] Enzyme concentration required to inhibit the chromogen produced by 50% in 1 min under standard conditions.
[b] Micromoles of H_2O_2 decomposed.
* $P < 0.001$ as compared with normal.

with normal subjects. Plasma conjugated dienes were also significantly increased in ovarian cancer patients as compared with normal subjects.

Table 2 shows the level of antioxidants in the circulation of normal and ovarian cancer patients. The enzymatic antioxidants such as SOD and CAT in the hemolysate were significantly lower in ovarian cancer patients as compared with normal subjects. Also the non-enzymatic antioxidants vitamins C and E in the plasma were significantly lower in ovarian cancer patients vs. controls.

4. Discussion

Oxidative stress is due to a disturbance in the balance between the production of ROS and the efficiency of the antioxidant defense. In other words, oxidative stress results if excessive production of ROS overwhelms the antioxidant defense system or when there is a significant decrease or lack of antioxidant defense [20]. Potential biological targets for free radical attack include lipids, proteins and nucleic acids [21]. The epoxides generated due to increased oxidative stress may spontaneously react with nucleophilic centers in the cell and thereby covalently bind to DNA, RNA and protein. Such reactions may lead to cytotoxicity and carcinogenicity depending on the properties of the epoxides [22]. Moreover, severe oxidative stress is not only known to cause DNA damage and mutations of tumor suppressor genes, which are initial events in carcinogenesis [20], but can also play an important role in the promotion of multistep carcinogenesis [23].

Lipids, especially polyunsaturated fatty acids (PUFA), are very susceptible to free radical attack, which can initiate lipid peroxidation [24]. Lipid peroxidation plays an important role in the control of cell division [25]. The end product of lipid peroxidation, malondialdehyde, due to its high cytotoxicity and inhibitory action on protective enzymes, is suggested to act as a tumor promoter and a co-carcinogenic agent [26]. An inverse relationship has been observed between lipid peroxidation and the rate of cell proliferation, with highly proliferating tumors showing low levels of lipid peroxidation [27]. Studies show that in contrast to decreased lipid peroxidation in tumor tissues, enhanced lipid peroxidation is observed in the circulation of cancer patients. In our earlier studies, we have observed increased lipid peroxidation and decreased antioxidant levels in the plasma of cervical cancer patients [28]. In our present work, we noticed increased levels of circulating TBARS and conjugated dienes in ovarian cancer patients which can be attributed to the deficiency of antioxidant defense mechanisms.

The antioxidant enzymes, SOD and CAT, widely distributed in all cells, are present in high amounts in erythrocytes [29]. SOD protects cells against $O_2^{-\cdot}$ by dismutation of the highly reactive superoxide anion to O_2 and to a less reactive species, H_2O_2 [30]. CAT, in turn, protects the cell from H_2O_2 generated by various reactions [31]. In our studies, we observed low levels of SOD and CAT in ovarian cancer patients. The observed increase in circulating lipid peroxides of ovarian cancer patients correlate with the decline in SOD and CAT activity. This can result in accumulation of superoxide anion, highly diffusible and potent oxidizing radical capable of traversing membranes, causing deleterious effects at sites far from the tumor [32]. A decrease in the activity of CAT could be due to increase in the lipid peroxidation product, malondialdehyde which can form cross links, thereby inactivating several membrane bound enzymes [33,34]. The increase in circulating lipid peroxides may be related to a deficiency of SOD in tumor tissues. Decreased CAT activity could also be due to exhaustion of the enzyme because of increased peroxidation.

Vitamin E is a lipid-soluble, chain terminator antioxidant present along with lipids in the cell membranes [35]. Vitamin C, a water-soluble, radical scavenging antioxidant [11], present in all cells can also act as a reducing agent. Vitamin C can neutralize vitamin E radical getting itself converted to a free radical (unreactive) while regenerating vitamin E [36]. A positive correlation between vitamins E and C deficiency and lipid peroxide formation has been documented [37]. In addition to its antioxidant potential, vitamin E also functions as a biologic response modifier influencing the production of second messengers and products of the arachidonic acid cascade which have profound effects on cell proliferation [38].

Epidemiology studies support an inverse relationship between circulating levels of vitamins C and E with ovarian cancer [39,40]. Due to low economic status, women were malnourished and thus were more

prone to malignancy. Studies indicate that women who consume fewer amounts of fruits and vegetables are associated with ovarian cancer [41]. Thus the enhanced lipid peroxidation observed in ovarian cancer patients can also be attributed to a large extent to the depletion of vitamins E and C in the diet. In our studies also we observed low levels of vitamins C and E in the circulation of ovarian cancer patients. The decreased levels of plasma vitamins E and C may be due to their increased utilization in scavenging lipid peroxides as well as sequestration by tumor cells [28].

Thus, low levels of SOD, CAT, vitamins C and E in the ovarian cancer patients may be due to increased utilization to scavenge lipid peroxides as well as their sequestration by tumor cells. Increased levels of lipid peroxidation may be due to excessive oxidative stress caused by incessant ovulation or epithelial inflammation initiators such as talc and asbestos.

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